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Journal of Microbiology, Immunology and Infection (2014) **47**, 447–454

Available online at www.sciencedirect.com

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ORIGINAL ARTICLE

Phylogenetic analysis and development of an immunofluorescence assay for untypeable strains of coxsackievirus B3

Yuan-Pin Huang [a,c], Tsuey-Li Lin [a,c], Yu-Ju Chen [a], Chiu-Chu Hsu [a], Ting-Han Lin [a], Ho-Sheng Wu [a,b,*]

[a] *Research and Diagnostic Center, Centers for Disease Control, Department of Health, Taipei, Taiwan, ROC*

[b] *School of Medical Laboratory Science and Biotechnology, Taipei Medical University, Taipei, Taiwan, ROC*

Received 15 April 2013; received in revised form 22 June 2013; accepted 10 July 2013
Available online 29 August 2013

KEYWORDS
Coxsackievirus B3;
Immunofluorescence assay;
Phylogenetic analysis;
Subgenogroup

Background/Purpose: In recent years, coxsackievirus B3 (CV-B3) has been determined as a dominant enterovirus serotype that may cause severe complications in patients. Since 2008 in Taiwan, some enterovirus isolates have been regarded as untypeable [by employing commercial immunofluorescence assay (IFA) kits]. In 2012, the number of isolates increased. Genetic sequence analysis further confirmed that CV-B3 was present in most of the untypeable viruses.

Methods: Isolates of CV-B3 were collected for basic local alignment search tool (BLAST) analysis and for phylogenetic analyses, based on *VP1* gene sequences. In addition, the Taiwan Centers for Disease Control (Taiwan CDC) developed an in-house indirect IFA using polyclonal antibodies (e.g., rabbit antisera) for diagnosis. The sensitivity and specificity were both evaluated by testing 61 reference enteroviruses and 307 local enteroviruses that were isolated between 1998 and 2010.

Results: Based on the results of the BLAST and phylogenetic analyses, five main genogroups (i.e., GI–GV) were classified and the reference strains in Taiwan in previous years were primarily clustered in the GV-A subgenogroup. However, the 15 CV-B3 isolates recently analyzed in this study were classified in four different groups: GIII, GIV, GV-A, and GV-B. Among these 15 isolates, all 10 isolates in the GV-B group were initially reported as untypeable nonpolio enteroviruses when using commercial kits. The conditions of the in-house indirect IFA were

* Corresponding author. Research and Diagnostic Center, Centers for Disease Control, Department of Health, Number 161, Kun-Yang Street, Taipei City 11561, Taiwan, ROC.
E-mail address: wuhs@cdc.gov.tw (H.-S. Wu).

[c] Y.-P. Huang and T.-L. Lin contributed equally to this work.

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http://dx.doi.org/10.1016/j.jmii.2013.07.003

optimized by checkerboard titration, thereby resulting in a sensitivity of 100% and a specificity of 98.5%.
Conclusion: This is the first report describing the phylogenetic relatedness of recent CV-B3 strains in Taiwan. An indirect IFA kit was developed by the Taiwan CDC for detecting CV-B3 viruses that are untypeable by commercial IFA kits.
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Introduction

Coxsackievirus B3 (CV-B3) is a member of the genus *Enterovirus* of the family *Picornaviridae*. The genus *Enterovirus*, which contains more than one hundred serotypes, was originally composed of polioviruses, coxsackievirus A, coxsackievirus B, and echoviruses on the basis of differences in infectivity, cell tropism, antigenicity, and pathogenicity.[1,2] However, in recent years, the genus has been reclassified (based on molecular characteristics) into 10 species: *Human enterovirus A*, *Human enterovirus B*, *Human enterovirus C*, *Human enterovirus D*, *Simian enterovirus A*, *Bovine enterovirus*, *Porcine enterovirus B*, *Human rhinovirus A*, *Human rhinovirus B*, and *Human rhinovirus C*. The CV-B3 and other 59 serotypes are now members of *Human enterovirus B*.[3] The picornavirus genome is organized into four sections: (1) the 5′ nontranslated region; (2) the single open reading frame encoding a polyprotein; (3) the 3′ nontranslated region; and (4) the poly(A) tail. The polyprotein includes four structural capsid proteins (i.e., VP4, VP2, VP3, and VP1) and seven nonstructural proteins (i.e., 2A, 2B, 2C, 3A, 3B, 3C, and 3D). Virus isolation is traditionally used for the diagnosis of enterovirus infection, whereas immunofluorescence assay (IFA) and the neutralization test are used for serotyping and antigenic characterization. Molecular diagnostic methods now offer a faster and more sensitive alternative to traditional immunoassays and culture techniques and the methods provide valuable information for genetic studies.[1] However, because molecular diagnosis requires expensive equipment and trained laboratory technicians, it is not yet widely used in most clinical laboratories. In Taiwan, the IFA is the method of choice to screen clinical specimens for enteroviruses in the front end of the surveillance system. According to our sentinel physician surveillance system, more than 10,000 specimens are collected every year, and more than 2000 enterovirus strains on average have been isolated.[4,5]

The VP1 protein contains several major neutralization antigenic sites and corresponds to its serotype, especially in the prominent exterior loops (e.g., the BC loop and the DE loop). Sequencing of the VP1 region has been used for genotyping and phylogenetic analysis, and five genogroups have been classified in CV-B3[1,2,6–8]; however, only few phylogenetic studies on CV-B3 are available.

Enteroviruses, particularly coxsackievirus B, are the most common agents of viral myocarditis and are associated with dilated cardiomyopathy.[9,10] In the years 1999, 2000, and 2005 in Taiwan, the CV-B3 was a very prevalent enterovirus and was associated with severe complications in some patients.[5,11] In 2008, some CV-B3 isolates were furthermore initially untypeable by commercial IFA kits, but they were finally confirmed by sequence analysis. Therefore, the purpose of this study is to determine if recent CV-B3 isolates are genetically and antigenically distinct from isolates of earlier outbreaks. The Taiwan Centers for Disease Control (Taiwan CDC) developed an in-house IFA kit to evaluate the untypeable CV-B3 viruses used in this study.

Materials and methods

Virus isolation and identification

Enteroviruses used in the study were collected and isolated from patients with symptoms related to enterovirus infection (Table 1).[5] For virus isolation, the Taiwan CDC Collaborating Laboratories of Virology (TCCLV) used several cell lines such as African green monkey kidney (Vero) cells, human diploid fibroblast (MRC-5) cells, human lung carcinoma (A549) cells, human epidermoid carcinoma (HEp-2) cells, monkey kidney (LLC-MK2) cells, and rhabdomyosarcoma (RD) cells. The isolates were identified by IFA using commercial monoclonal antibodies (Light Diagnostics, Millipore Corporation, Billerica, MA, USA) and by the in-house kit provided by the Taiwan CDC. These isolates, which included nontypeable strains, were sent on a regular monthly basis to the Taiwan CDC for further genotyping.

Viral RNA extraction, reverse transcription-polymerase chain reaction, and sequencing

Viral RNA was extracted from 140 μL of culture supernatant in accordance with the manufacturer's instructions by using the QIAamp Viral RNA Mini Kit (Qiagen, Santa Clara, CA, USA). Extracted RNA was used in a one-step reverse transcription-polymerase chain reaction (RT-PCR) of the *VP1* gene, as described previously.[12,13] The products were sequenced in both directions by using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an automated sequencer (ABI 3730; Applied Biosystems, Foster City, CA, USA).

Phylogenetic analysis and selection analysis

By using BLAST software (National Center for Biotechnology Information, Bethesda, MD, USA), genotyping was performed by comparing the obtained sequences with sequences in the GenBank. The genotyping was then

Table 1 Yearly distribution of nonpolio enterovirus isolates[a]

Year	No. of NPEV isolates	No. of untypeable NPEV isolates[b]	No. of CV-B3 isolates[c]	
			Reported as untypeable NPEV	Reported as NPEV
2007	2456	472	0	1
2008	4027	368	6	2
2009	2423	296	0	0
2010	3419	155	2	0
2011	3425	247	3	0
2012	2367	192	35[d]	83
Total	18,117	1730	46	86

[a] The EV isolates analyzed in this study were collected by the surveillance systems under the Taiwan Centers for Disease Control. The number was counted on the basis of the amount of received isolates. The isolates were excluded from this study if the patient information was incomplete.
[b] The isolates could not be identified by the immunofluorescence assay (IFA) kit.
[c] The information is based on genotyping results.
[d] Prior to the use of the in-house IFA kit, developed in May 2012, most CV-B3 isolates in 2012 were untypeable by IFA.
NPEV = nonpolio enterovirus.

confirmed by phylogenetic analysis, based on the *VP1* gene[8] by using the Molecular Evolutionary Genetics Analysis 5.03 (MEGA 5.03) software.[14,15] The sequences determined in the study were compared with reference sequences obtained from GenBank and from previous surveillance results. The phylogenetic tree combined with a proteotyping map using complete *VP1* gene sequences was furthermore performed to compare sequence differences. Phylogenetic trees were constructed by using the neighbor-joining method with 1000 replicates of bootstrap resampling. Amino acid substitutions were visualized through a proteotyping map. Different amino acids were indicated by single letter abbreviations in different colors. The amino acid residues of each virus were presented on the *x* axis on the map, and each virus was ordered according to the phylogenetic tree on the *y* axis. Each column represented the amino acid position, indicated by a selection criterion of 5%. The sequences were edited by using BioEdit software (Ibis Biosciences, Carlsbad, CA, USA).[16] Nonsynonymous (dN) and synonymous (dS) nucleotide substitutions were used to examine the positive selection on the Datamonkey webserver.[17,18] A positive selection was defined as a dN value greater than the dS value with $p < 1.0$. Three methods were used to detect the site selection: (1) single-likelihood ancestor counting (SLAC); (2) fixed effects likelihood (FEL); and (3) internal FEL (IFEL).[18,19]

Production of polyclonal antibodies

The CV-B3 strain E2005351, which was isolated in Taiwan, was used as the immunogen for the antiserum preparation. The strain was propagated in the RD cells and the $10^{7.3}$/50 μL 50% cell culture infectious dose ($CCID_{50}$), calculated by the Reed and Muench method,[20] was determined before administering the rabbit vaccinations. Anti-CV-B3 rabbit serum was prepared, as described previously.[21] In brief, four New Zealand white rabbits were intravenously immunized five times in a 2-day interval with 5 mL of UV-inactivated virus stock. The rabbits received a final booster of 10 mL of the virus stock on Day 42, and their sera were tested for neutralization antibodies on Day 49. The care and use of the rabbits were approved by the Institutional Animal Care and Use Committee of The Taiwan CDC to ensure compliance with the local legal and ethical requirements.

Determination of neutralization antibody titers

After inactivation at 56°C for 30 minutes, the rabbit antisera were two-fold serially diluted in Dulbecco's modified Eagle medium (DMEM), and then examined for

Table 2 Prototype strains and clinical isolates of HEV and other pathogens used for the determination of sensitivity and specificity by using rabbit antiserum against coxsackievirus B3

	Prototype strains ($n = 61$)[a]	Clinical isolates ($n = 307$)
HEV-A	CV-A2 to CV-A8, CV-A10, CV-A12, CV-A14, CV-A16, enterovirus A71 (EV-A71)	CV-A2 (16 isolates), CV-A3 (25 isolates), CV-A4 (25 isolates), CV-A5 (9 isolates), CV-A6 (9 isolates), CV-A8 (5 isolates), CV-A10 (10 isolates), CV-A12 (5 isolates), CV-A16 (8 isolates), EV-A71 (83 isolates)
HEV-B	CV-A9, CV-B1, CV-B2, CV-B4 to CV-B6, echovirus 1 (E-1) to E-7, E-9, E-11 to E-21, E-24 to E-27, E-29 to E-33, EV-B69, EV-B73, CV-B3	E-3 (10 isolates), E-4 (2 isolates), E-6, E-7, E-11, E-16 (4 isolates), E-18 (6 isolates), E-25 (10 isolates), E-30 (8 isolates), E-33, CV-B1, CV-B2 (3 isolates), CV-B4 (3 isolates), CV-B5, CV-B3 (42 isolates)
HEV-C	CV-A11, CV-A13, CV-A17, CV-A18, CV-A21, poliovirus 1 to poliovirus 3 (vaccine strain)	CV-A21 (6 isolates)
HEV-D	EV-D68, EV-D70	EV-D68 (5 isolates)
Others	Parechovirus 1, parechovirus 2	Herpes simplex virus (5 isolates), influenza virus, rhinovirus 31

[a] Unless specifically indicated, only one strain is used.
E = echovirus; EV = enterovirus; HEV = human enterovirus.

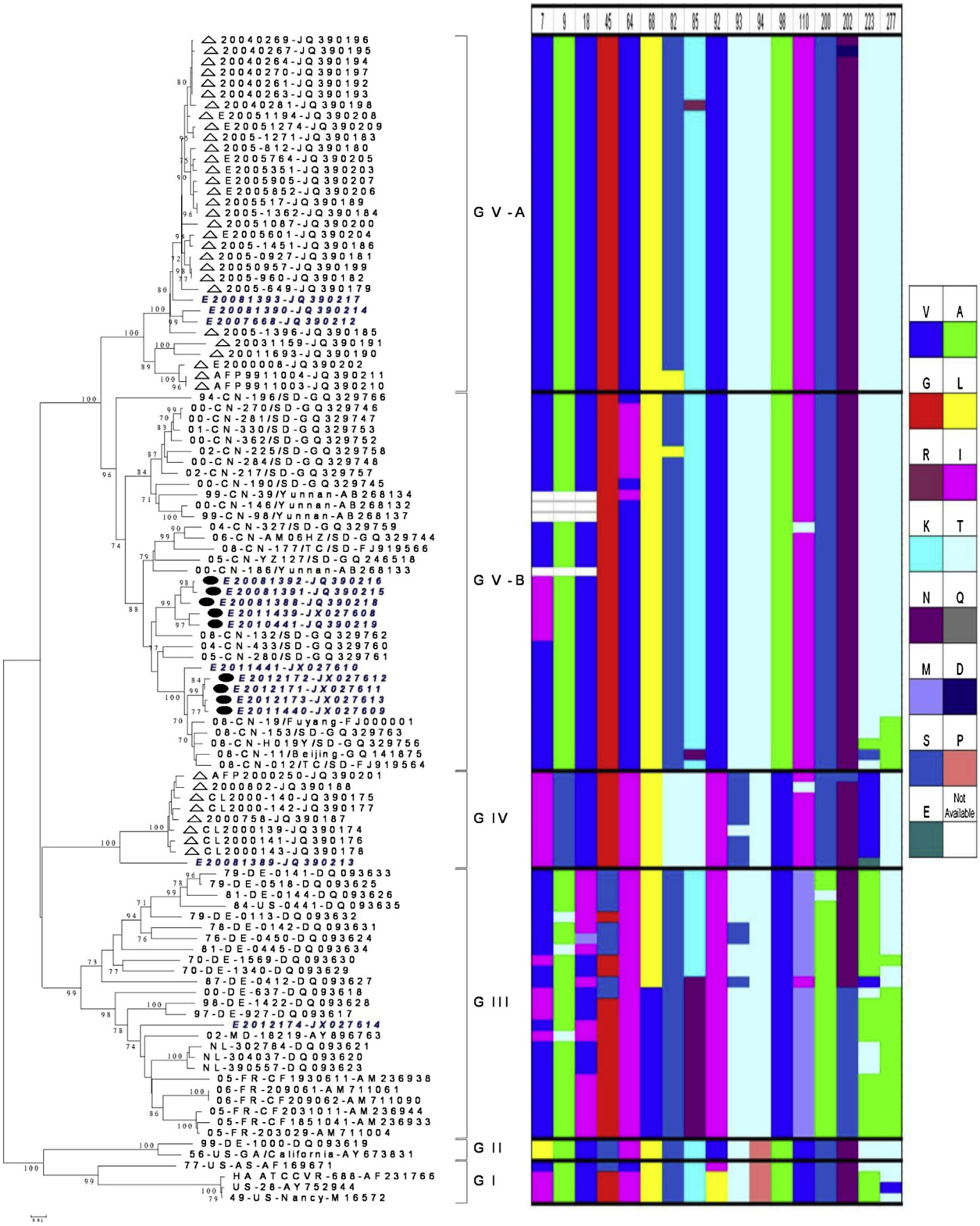

Figure 1. Phylogenetic analysis of the coxsackievirus B3 strains, based on the complete *VP1* gene sequence (852 nucleotides). Phylogenetic analysis is based on the complete *VP1* gene nucleotide sequences of reference strains from GenBank, from 38 Taiwan CV-B3 isolates previously obtained by the surveillance system from 1999 through 2005, and from 15 representative Taiwan isolates from 2007 through 2012. The phylogenetic tree was constructed by using the Molecular Evolutionary Genetics Analysis (MEGA, version 5) software, using the neighbor-joining method. The reliabilities were evaluated by using 1000 bootstrap replications. Only bootstrap values of over 70% are shown. The 15 isolates from 2007 through 2012 are represented in italic boldface. The black dots indicate isolates that were untypeable by the commercial IFA kit. The white triangles indicate the 38 isolates from 1999 through 2005. The reference

neutralization antibodies. In a carbon dioxide incubator, 50 μL of 100 $CCID_{50}$ viruses of different CV-B3 subgenogroups and 50 μL of the aforementioned serially diluted antiserum were incubated at 36°C for 60 minutes. A RD cell suspension (100 μL) containing approximately 5×10^4 cells was added afterward to each well. The cytopathic effects (CPE) were observed daily during the following 4 days. Neutralization titers are expressed as the reciprocal of the highest serum dilution at which 50% of the cells represent complete inhibition of the formation of CPE.

Indirect IFA and optimization of antiserum dilution

The procedure for IFA staining has been described previously.[21] In brief, the rabbit antiserum is adequately pipetted to cover each testing well on a glass slide. After incubation for 30 minutes at 37°C, the slide is washed with phosphate-buffered saline (pH 7.2—7.4, 0.05% Tween 20) and then dried. Ten microliters of fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (i.e., secondary antibody) is added to each well and incubated for 30 minutes at 37°C. After the slide is washed and air dried, the fluorescence is observed. A positive result was indicated by an apple-green color in the cytoplasm and nucleus of infected cells. The optimization of antiserum dilution was performed by using the serial dilution method.

Determination of sensitivity and specificity

The pretest was performed by using 61 prototype strains, which consisted of human enterovirus A (HEV-A), HEV-B, HEV-C, HEV-D, parechovirus 1, and parechovirus 2. Between 1998 and 2010, a total of 307 clinical isolates collected in Taiwan were used for determining sensitivity and specificity (Table 2). All tested isolates have been preidentified for their serotypes or genotypes by RT-PCR and sequencing.

Statistical analysis

The neutralization antibody titers were compared between the GV-A subgenogroup and other clusters by using the Student t test with $\log_{10}$-transformed data. A $p < 0.05$ indicated statistical significance. The sensitivity and specificity and the 95% confidence interval values were calculated by using a web-based program (http://faculty.vassar.edu/lowry/clin1.html). The kappa value and the 95% confidence interval values between the indirect IFA method and the molecular identification were calculated by using a web-based program (http://faculty.vassar.edu/lowry/kappa.html).

Nucleotide sequence accession numbers

The 53 nucleotide sequences of the *VP1* gene determined in this study have been deposited in the GenBank database under the following IDs: JQ390174—JQ390219 and JX027608—JX027614.

Results

Epidemiological results

The enterovirus isolates used in the study are listed in Table 1. After RT-PCR and sequencing, the *VP1* gene sequences obtained from these viruses were used for genotyping. Based on the BLAST genotyping data, there was one CV-B3 isolate in 2007; eight CV-B3 isolates in 2008; zero CV-B3 isolates in 2009; two CV-B3 isolates in 2010; three CV-B3 isolates in 2011; and 118 CV-B3 isolates in 2012 (Table 1). An obvious increase in CV-B3 infection was also detected in early 2012. Six of the eight isolates in 2008, two isolates in 2010, three isolates in 2011, and most isolates in early 2012 were reported as untypeable nonpolio enteroviruses (NPEV) by IFA using commercial monoclonal antibody (Light Diagnostics, Millipore Corporation, Billerica, MA, USA).

Phylogenetic analysis and selection analysis

For phylogenetic analysis, the following were used: the 15 Taiwan CV-B3 isolates in this study that were collected from 2007 through 2012 (one strain was isolated in 2007 and in 2010; six strains in 2008; three strains in 2011, and four strains in 2012) and 38 Taiwan CV-B3 isolates randomly chosen from the reference isolates from 1999 through 2005. According to the phylogenetic tree (Fig. 1), five main genogroups were classified (i.e., GI—GV), and the strains isolated in Taiwan between 1999 and 2005 were primarily clustered in the GV-A subgenogroup. The 15 isolates obtained between 2007 and 2012 were separated into four different subgenogroups, as follows: one strain each in GIII and in GIV; three strains in GV-A; and ten strains in GV-B. In GV-B, all 10 isolates (i.e., E20081388, E20081391, E20081392, E2010441, E2011439, E2011440, E2012173, E2012172, E2012171, and E2011441) were interestingly reported as untypeable NPEV. One unique amino acid substitution at position 7 (valine to isoleucine) was observed in 5 of 10 isolates in subgenogroup GV-B when compared to the isolates in subgenogroup GV-A (Fig. 1). No amino acid residue under positive selection was identified.

Preparation of antienterovirus rabbit serum and results of the neutralization test

Four anti-CV-B3 rabbit sera (#95, #96, #97, and #98) against the GV-A subgenogroup (the predominant subgenogroup in Taiwan) were used for the neutralization test. Table 3 shows the neutralization antibody titers against different subgenogroups of CV-B3. There was an 8- to 64-fold decrease in titers against the hetero-subgenogroup virus

sequences are labeled in the order of year and abbreviated country name of the coxsackievirus B3 strain-GenBank accession number (i.e., CN = China; FR = France; DE = Germany; MD = Republic of Moldova; NL = The Netherlands; US = United States). Amino acid substitutions were visualized through a proteotyping map. Different amino acids are indicated by single-letter abbreviations in different colors. Each column represents the amino acid position, indicated with a selection criterion of 5%.

Table 3 Neutralization antibody titers of rabbit antisera against coxsackievirus B3 from different subgenogroups

Subgenogroup of the CV-B3 virus (strain)	Antiserum no.			
	#95	#96	#97	#98
GV-A (E2005351)[a]	16,384	32,768	65,536	16,384
GIV* (E2000250)	1024	1024	1024	256
GV-B* (E20081391)	2048	512	1024	256

[a] The GV-A subgenogroup strain E2005351, which was isolated in Taiwan, was used as the immunogen for the antiserum preparation.
*Indicates a statistically significant difference in the $\log_{10}$-transformed data, compared to the GV-A subgenogroup ($p < 0.05$).
CV-B3 = coxsackievirus B3.

($p < 0.05$). Based on the neutralization test results, antiserum #95 was selected for the indirect IFA.

Indirect IFA and the determination of sensitivity and specificity

The in-house antiserum #95 and three monoclonal antibodies [i.e., mAbs catalog number 3360 of the pan-enterovirus (pan-EV) blend, catalog number 3303 of a CV-B blend, and catalog number 3306 of CV-B3] from the commercial kit were used for the detection of different genogroups among the CV-B3 isolates (Fig. 2). The isolate E2005351 of the GV-A subgenogroup and the isolate AFP2000250 of the GIV group were positive for all four antibodies (panels A—D and panels E—H, respectively, in Fig. 2). By contrast, the isolate E20081391 of the subgenogroup GV-B was negative for CV-B mAb (catalog numbers 3303 and 3306), but positive for pan-EV mAb (catalog number 3360) and antiserum #95 (panels I—L in Fig. 2). The sensitivity of the indirect IFA was 100.0% (95% confidence interval, 89.8—100.0%), as evaluated by using 43 CV-B3 viruses (i.e., one prototype strain and 42 clinical isolates). The specificity was 98.5% (95% confidence interval, 96.2—99.4%), calculated by using 325 non-CV-B3 isolates (i.e., 60 prototype strains and 265 clinical isolates; Table 2). The kappa value was 0.94 (95% confidence interval, 0.88—0.99), indicating high reproducibility between the indirect IFA method and molecular identification.

Discussion

Enterovirus infections are common in Taiwan. Hence, a rapid and sensitive method is required for identifying the serotype of an enterovirus. The neutralization test is the gold standard for typing enteroviruses, but it is more complicated and time consuming. The indirect IFA therefore has become a very common method that is used in clinical diagnostics. Because commercial IFA kits now identify only 19 enterovirus serotypes (i.e., poliovirus 1 to poliovirus 3, CV-B1—CV-B6, CV-A9, CV-A16, CV-A24, E-4, E-6, E-9, E-11, E-30, EV-D70, and EV-A71), the Taiwan CDC has developed an indirect IFA kit for the serotyping of enteroviruses CV-A2, CV-A4, CV-A5, CV-A6, and CV-A10, which have been circulating in Taiwan in recent years. The kit has effectively reduced the quantity of untypeable enteroviruses in clinical virology laboratories.[21] In the past, the CV-B3 virus could be identified by commercial IFA kits, except for some isolates in Hong Kong.[22] The IFA originally reported several isolates as "untypeable". However, since 2008 in Taiwan, the isolates have been finally confirmed as CV-B3 by sequencing and neutralization tests.

CV-B3 was one of the most prevalent types of virus in confirmed severe EV cases.[5] Based on a previous study, five genogroups are identified among the CV-B3 strains.[8] This study analyzed more than 100 VP1 nucleotide sequences in CV-B3 strains isolated worldwide. Fifteen Taiwan isolates were separated in four different subgenogroups (i.e., GV-A, GV-B, GIII, and GIV). All 10 isolates in the GV-B subgenogroup interestingly were reported as "untypeable NPEV" in the study, indicating that mutations in GV-B subgenogroup may contribute to the untypeable characteristic. However, only one amino acid substitution at position 7 (i.e., valine to isoleucine) was observed on comparing these isolates with other typeable Taiwan isolates in 2008. The same amino acid substitution at the same position was also present in the GIV strains, which first emerged in Taiwan in 2000 and then disappeared quickly. However, the difference of the IFA result was present between the old GIV strains and the re-appeared isolate in 2008 (i.e., E20081389); the latter isolate was typeable by a commercial IFA kit. Hence, more strains and studies are needed to clarify the correlation between the amino acid substitution and the untypeable characteristic. In addition, the amino acid substitution at position 277 (i.e., threonine to alanine) observed in some isolates in Hong Kong was possibly associated with central nervous system disease.[22] However, this substitution was not displayed in these Taiwan isolates. By contrast, there was one Taiwan isolate (i.e., E2012174) in the GIII subgenogroup that contained older strains from the 1970s to 1980s and some strains in France from 2005 to 2006, but it is not easy to trace back the actual spread route because of frequent international travel and trade activities. An increase in CV-B3 infection furthermore occurred in early 2012, and most isolates were reportedly untypeable NPEV. This indicates that a useful diagnostic tool is needed for the surveillance system, and that a potential CV-B3 outbreak cannot be ignored.

The CV-B3 virus is a very common enterovirus serotype circulating in Taiwan.[11] In 2005, the clinical symptoms caused by the CV-B3 virus were more complicated and resulted in the deaths of several neonates in Taiwan. The virus characteristics and herd immunity may have accounted for the CV-B3 recurrence in 1999—2000 and in 2005, which included susceptible children and neonates. Viral evolution may also influence circulation, and a new imported subgenogroup or some new variants could cause a new epidemic. For example, in 2008 the B5 subgenogroup of EV-71 caused serious outbreaks in Taiwan.[23] Because of the potential for severe and fatal outbreaks, it is necessary to monitor carefully the antigenic and genetic evolution of the CV-B3 virus.

With the exception of the false-positive results with herpes simplex virus (HSV), good sensitivity and specificity were observed by the indirect IFA using rabbit antiserum in this study. The same cross-reactivity with HSV was obtained

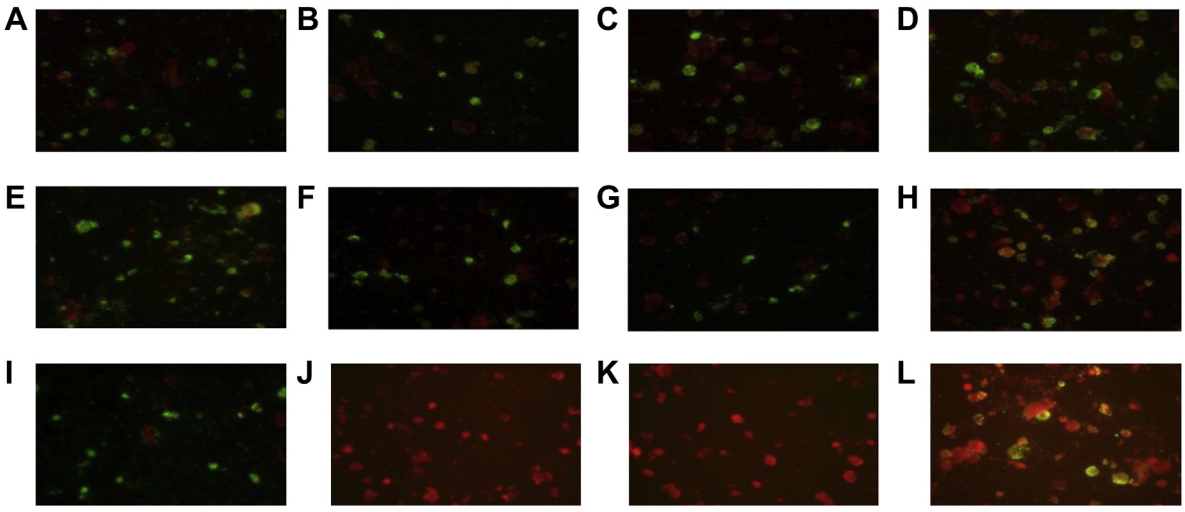

Figure 2. Photographs of IFA staining using an in-house IFA kit. The rhabdomyosarcoma (RD) cells infected with the coxsackievirus B3 (CV-B3) isolates are stained and show positive apple-green fluorescence with the commercial IFA kit and the in-house IFA kit (400× magnification). Three CV-B3 genogroups were used: isolate E2005351 in genogroup GV-A (panels A, B, C, and D); isolate AFP2000250 in genogroup GIV (panels E, F, G, and H); and isolate E20081391 in genogroup GV-B (panels I, J, K, and L). The CV-B3 isolates were stained by monoclonal antibody catalog number 3360 of the Pan-Enterovirus Blend (panels A, E, and I); catalog number 3303 of the CV-B blend (panels B, F, and J); catalog number 3306 of CV-B3 (panels C, G, and K); and antiserum #95 (panels D, H, and L). However, the isolate E20081391 was untypeable by catalog numbers 3303 and 3306.

when using different sources of rabbit sera before and after immunization (data not shown), which demonstrates that unknown components in rabbit serum may induce a nonspecific reaction with HSV in the IFA method. A previous study also reported cross-reactivity between HSV and rabbit antiserum in the IFA kit.[21] The false-positive result caused by HSV could nevertheless be distinguished by different cytopathic effects or by HSV-specific IFA.

In summary, the phylogenetic relatedness of the CV-B3 strains (isolated in Taiwan from 2007 through 2012) was described and may help in understanding the evolution and diversity of the virus. Some strains were untypeable by using the commercial IFA kit and were classified in the GV-B sugbenogroup. An indirect IFA kit for the detection of CV-B3 was developed. This kit was evaluated at The Taiwan CDC and test results showed very good sensitivity (100%) and specificity (98.5%). Because of the rapid evolution of enteroviruses and the potential of re-emerging pathogens and severe outbreaks, a regular surveillance system and continually improved diagnostic methods are needed.

Conflicts of interest

The authors declare that they have no conflicts of interest related to the work in this article.

Acknowledgments

The authors greatly appreciate the chiefs of the Taiwan Centers for Disease Control (CDC) Collaborating Laboratories of Virology for their cooperation in making this study possible. They are Chuan-Liang Kao, Jang-Jih Lu, Yu-Jiun Chan, Kuo-Chien Tsao, Ming-Jer Ding, Mu-Chin Shih, Chi-Ho Chan, Jen-Shiou Lin, Jen-Ren Wang, Kuei-Hsiang Lin, Yung-Ching Liu, Hock-Liew Eng, Li-Kuang Chen, Tzong-Shi Chiueh, Sui-Yuan Chang, Shin-Ru Shih, John Wang, and Yao-Shen Chen. This study was supported financially by research grants from The Taiwan CDC and the National Research Program for Genomic Medicine. The findings and conclusions in this study are those of the authors and do not necessarily represent the views of the Taiwan CDC.

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